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Different expression of the plasminogen activation system in renal thrombotic microangiopathy and the normal human kidney

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Different expression of the plasminogen activation system in renal thrombotic microangiopathy and the normal human kidney. Renal thrombotic microangiopathy is characterized by glomerular and vascular thrombosis. The persistancy of fibrin deposits may result from imbalance between plasminogen activation and inhibition. In the present study, we used immunohistochemistry and *in situ* hybridization techniques to determine the localization of urokinase-type (u-PA) and tissue-type (t-PA) plasminogen activators, type 1 plasminogen activator inhibitor (PAI-1) and membrane receptor for u-PA (uPA-R) antigen and their sites of synthesis in renal thrombotic microangiopathy ($N = 10$) as compared to acute tubular necrosis ($N = 5$) and normal human kidneys ($N = 7$). We found an induction of PAI-1 and uPA-R expression in glomeruli and in arterial walls in renal thrombotic microangiopathy. In addition, the induction of uPA-R expression was also found in some tubular epithelial cells. In most cases, local synthesis of PAI-1 and u-PA-R was confirmed by *in situ* hybridization with the corresponding cDNA probes. In contrast, using similar techniques PAI-1 and uPA-R antigens and messenger RNAs could not be detected in normal kidneys. In both renal thrombotic microangiopathy and normal kidneys, t-PA mRNA was detected in large amounts in all glomeruli and in vascular endothelial cells, but t-PA antigen was only detected in a limited number of glomerular and arterial endothelial cells, whereas it was strongly expressed by all venous endothelial cells. Although u-PA antigen was found in almost all tubular sections, u-PA mRNA was only found in tubular epithelial cells in the deep cortex and the outer medulla. Our results indicate that there is an up-regulation of PAI-1 and u-PA-R expression in the glomeruli and in the arterial walls of thrombotic microangiopathy. The local release of PAI-1 could play a role in the persistancy of fibrin deposition and the further development of fibrotic lesions. Whether uPA-R plays a pathogenic role in the development of glomerular and vascular lesions, or is involved in the repair process of these lesions, remains to be elucidated.

Tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activators are two serine proteases that convert plasminogen into plasmin, a trypsin-like serine protease of broad specificity, involved in fibrinolysis [1] and extracellular matrix remodeling [2]. The binding of u-PA to its high affinity receptor (uPA-R) at the cell surface accelerates the activation of plasminogen into plasmin, which in turn activates pro-u-PA to u-PA [3, 4]. Therefore, uPA-R plays a crucial role for localizing u-PA at the cell surface and for local plasmin formation. t-PA, which is produced by endothelial cells, seems to be involved mainly in intravascular thrombolysis, whereas u-PA is known to be implicated in the extracellular matrix degradation [1, 2]. The activity of both t-PA and u-PA is controlled by plasminogen activator inhibitors of which PAI-1 appears to be the principal inhibitor [1].

In the murine kidney, using *in situ* zymography and *in situ* hybridization methods, Sappino et al demonstrated that t-PA is expressed in glomeruli and distal collecting ducts whereas u-PA is produced by epithelial cells of the straight part of proximal and distal tubules in the outer strip of the outer medulla [5]. A more diffuse distribution of u-PA antigen was demonstrated by immunohistochemistry, in epithelial cells of the medullary part of renal tubules [6]. It has also been demonstrated that PAI-1 and uPA-R are not detectable in the normal murine kidney but can be induced after endotoxin administration [7, 8]. In the rat kidney, PAI-1 up-regulation has been reported after injection of nephrotoxic serum [9, 10] or of Habu snake venom [11]. In the human kidney, we previously reported that t-PA antigen is detectable by indirect immunofluorescence in normal glomeruli and in endothelial cells of renal arterioles [12], and Hasui et al showed that u-PA antigen can be detected in almost all tubular sections [13]. To date PAI-1 expression in the normal human kidney has not been reported. However, we previously reported that PAI-1 antigen can be detected in fibrin deposits during renal thrombotic microangiopathy and severe extracapillary glomerulonephritis [14], and Wang et al showed that PAI-1 is detected in the endothelial cells of arterioles and arteries and also in infiltrating cells of transplanted kidneys with severe vascular rejection [15]. Finally, to our knowledge uPA-R expression in the human kidney has not been studied.

Thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS), two syndromes with different clinical presentations are characterized by a similar pathological lesion named thrombotic microangiopathy [16]. Fibrin deposition in glomerular capillaries and small arteries and/or arterioles is the main early finding. At a later stage, glomerular and arterial fibro-proliferative lesions can be observed [16]. The mechanisms of these lesions are unknown, but PAI-1 accumulation may be implicated, leading to a decrease of both fibrinolysis and extracellular matrix degradation. Although an elevated plasma PAI-1 level in patients with TTP [17] and HUS [18] has been reported, the origin of PAI-1 accumulated in glomeruli and renal vessels of these patients [14] remains to be determined, since PAI-1 may be

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released from endothelial cells of extrarenal vessels [19], aggregated platelets [20], or glomerular and renal vascular cells. Furthermore, it is not known if u-PA and uPA-R expressions are modulated in thrombotic microangiophathy, although they may be involved in angiogenesis, tissue degradation and repair [2].

To answer these questions, we used immunohistochemical and *in situ* hybridization techniques to determine the distribution of t-PA, u-PA, uPA-R and PAI-1 and their sites of synthesis in the kidney of patients with thrombotic microangiopathy as compared to those of patients with acute tubular necrosis and of normal human controls.

Methods

Materials

Thrombotic microangiopathy samples and normal human kidney. Thrombotic microangiopathy samples were obtained by percutaneous renal biopsies from patients with HUS/TTP and biopsy-proven thrombotic microangiopathy ($N = 10$). All patients (5 women, 5 men, median age of 37 years, range 19- to 58-years-old) had a clinical TTP/HUS presentation following an acute diarrhea ($N = 7$) which was hemorragic in three cases. The renal biopsy was performed from 5 to 15 days after the occurrence of the first symptoms, except in one case which was performed at day 45 when the patient was referred to our department for a recurrence of hemolysis and renal failure. The mean plasma creatinine level at the time of biopsy was 580 μmol/liter (range 180 to 1430). Except in one case, no patient had received fresh frozen plasma, nor steroids before the renal biopsy. The pathological diagnosis was performed by conventional light and immunofluorescence microscopy, and nine patients had predominant glomerular lesions and one patient had predominant vascular lesions. In six cases, tubular lesions were also observed. As the control with tubular lesions but without glomerular involvment, we used the biopsies of five patients with acute tubular necrosis. Normal human kidney specimens were obtained from the normal part of kidneys removed by nephrectomy for renal cell carcinoma ($N = 7$); the samples used were free of tumoral contamination by light microscopy. All samples were frozen in liquid nitrogen immediately after biopsy and/or nephrectomy without prefixation for immunohistochemical and *in situ* hybridization studies.

Reagents. The following antibodies were used: MuK4, a monoclonal anti-human u-PA antibody (Biopool, final concentration 5 μg/ml); PAM 3, a monoclonal anti-human t-PA antibody (Biopool, final concentration 10 μg/ml) and MAI 12, a monoclonal anti-human PAI-1 antibody (Monozyme Aps, final concentration 5 μg/ml). To analyze uPA-R expression, two monoclonal anti-uPA-R antibodies (R2, R4) previously characterized and shown to recognize different specific domains of uPA-R [21] and a polyclonal antibody against human uPA-R were used as previously described [22]. Secondary antibodies and visualization reagents were from Dako, Denmark; Sigma, USA; Amersham, UK and Vector, USA.

The probes used were a 1,000-bp *Pst*I insert of human PAI-1 cDNA (provided by Dr. David Loskutoff, Scripps Clinic and Research Foundation, La Jolla, CA, USA), a *Bgl*II fragment of the pPA 11 4B cDNA harboring 1948 bp of the human t-PA cDNA, a 1200 bp *Bam*HI fragment of human uPA-R cDNA (provided by E.K.O. Kruithof, CHUV, Lausanne, Switzerland) and a 600 bp *Eco*RI fragment of human u-PA cDNA (provided by R.L. Medcalf, CHUV, Lausanne, Switzerland), as previously described [23, 24]. The following reagents were used for *in situ* hybridization studies: Random Primers DNA-Labeling Kit from Bethesda Research Laboratory, USA; ^{35}S-dCTP from ICN, Costa Mesa, CA, USA.

Immunohistochemical studies

The fixation of anti-t-PA, anti-u-PA, anti-uPA-R (R2, R4) monoclonal antibodies was detected and visualized by the alkaline phosphatase anti-alkaline phosphatase complex technique (APAAP) (Dakopatt) according to previously described methods [25]. In the negative control, the primary antibody was omitted or replaced by normal mouse IgG1 (10 μg/ml) from Dako.

The fixation of polyclonal anti-uPA-R and of monoclonal anti-PAI-1 antibodies was detected and visualized by the biotin-streptavidin-peroxydase coupled technique. In brief, the tissue sections were first incubated with the specific rabbit polyclonal anti-u-PAR or mouse monoclonal anti-PAI-1 antibody, and after washing incubated with biotinylated anti-rabbit or anti-mouse antibody (Vector). Then the tissue sections were incubated with streptavidin coupled to peroxydase (Amersham, UK) which was visualized by 3,3′-diaminobenzidine (DAB) in the presence of H_2O_2 [26]. Sections were then counterstained with hematoxylin. To decrease the background due to streptavidin binding to endogenous biotin, tissue sections were preincubated with purified avidin and then with biotin before the incubation with the first antibody as previously described [27]. The intensity of immunohistochemical staining of uPA-R, PAI-1, t-PA and u-PA in the different renal structures (glomeruli, vessels, tubules) was assessed independently by two observers and semiquantitatively graded as −, +, ++ and +++ for no staining, light, moderate and strong staining, respectively, in comparaison with normal human kidney sections.

In situ hybridization studies

In situ hybridization was performed using ^{35}S-labeled cDNA probes as previously described [28]. The cDNA probes were labeled by ^{35}S-dCTP incorporation using the random primer method. Slides were first rinsed in alcohol, dried at room temperature, baked at 180°C for two hours and stored dust-free at room temperature. Cryostat tissue sections (8 μm) were put on uncoated slides and fixed in 4% formaldehyde in PBS for 10 minutes, rinsed in PBS and then dehydrated in graded alcohols and air-dried. Slides were stored at −20°C until analyzed (less than 1 month). Hybridization was carried out without predigestion in the mixture containing 0.2 ng/ml ^{35}S-labeled probe plus 4 × SSC (NaCl 3 M, trisodium citrate 0.3 M for 20 × SSC), 1 × Denhart (2% bovine serum albumin, 2% Ficoll, 2% polyvinylpyrrolidone for 100 × Denhart), 50% desionized formamide, 250 μg/ml yeast RNA, 500 μg/ml salmon sperm DNA and 5% dextran and DL-Dithiothreitol. After denaturation of the cDNA probe by heating at 100°C for five minutes, 10 μl of hybridization solution was applied to each section and then the sections were covered by siliconed coverslips and incubated overnight at 45°C in a moist chamber. After the hybridization, the sections were successively washed in 40% formamide 4 × SSC at 45°C, and then 2 × SSC at 60°C, 1 × SSC, 0.5 × SSC and 0.2 × SSC at room temprature for a total of four hours. The sections were dehydrated in graded alcohols and air-dried. Then the sections were dipped in 50% NTB2 emulsion (Eastman Kodak, Rochester, NY, USA) in water and exposed in a black box at 4°C for 14 days. The autoradiograph was developed in Kodak D19 for three minutes, fixed in Kodak

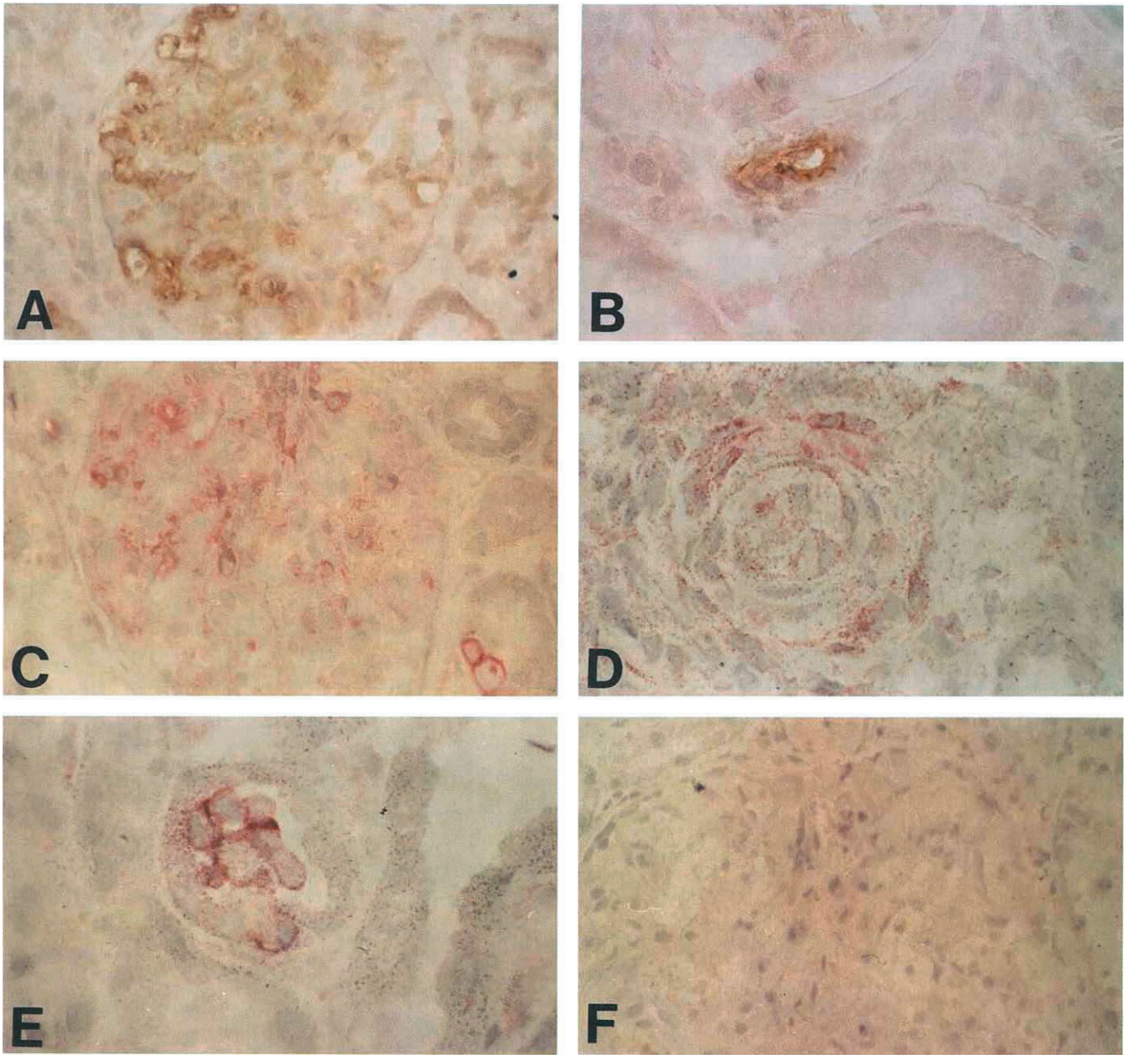

Fig. 1. *Localization of PAI-1 and uPA-R antigens in renal thrombotic microangiopathy by immunohistochemical studies (original magnifications: A, C, F 10 × 25; B, D, E 10 × 50).* By biotin-streptavidin-peroxydase coupled technique, a strong PAI-1 staining was observed in glomeruli (**A**) and in arterial endothelial cells (**B**) of renal thrombotic microangiopathy. uPA-R was also detected, by APAAP technique, in glomeruli (**C**), in vascular walls (**D**) and in tubular epithelial cells (C and E) especially on the surface of some desquamated epithelial cells (**E**). Incubation with normal mouse IgG1 as primary antibody showed no staining (**F**). Publication of this figure in color was made possible by a grant from Produits Roche, Neuilly-sur-Seine, France.

"rapid fix" and counterstained with hematoxylin. The negative controls were performed by preincubation of the sections with 100 μg/ml ribonuclease A (RNase) before the incubation with ^{35}S-labeled cDNA probe or incubation without the ^{35}S-labeled probe.

Results

Immunohistochemical studies

As shown in Figures 1 and 2 and in Table 1, with anti-PAI-1 antibody, an intense staining was found in glomeruli and in arterial walls, especially in endothelial cells in 7 out of 10 cases of thrombotic microangiopathy. No PAI-1 was detected in normal human kidneys nor in the cases of acute tubular necrosis. Similarly, a strong uPA-R expression was observed in glomeruli and in arterial walls of all thrombotic microangiopathy cases. In addition, an intense membrane uPA-R expression was also found on some tubular epithelial cells, particularly at the surface of desquamating epithelial cells. A similar tubular uPA-R expression was observed in the five cases of acute tubular necrosis. In these cases, uPA-R was only found at the surface of tubular epithelial cells, especially

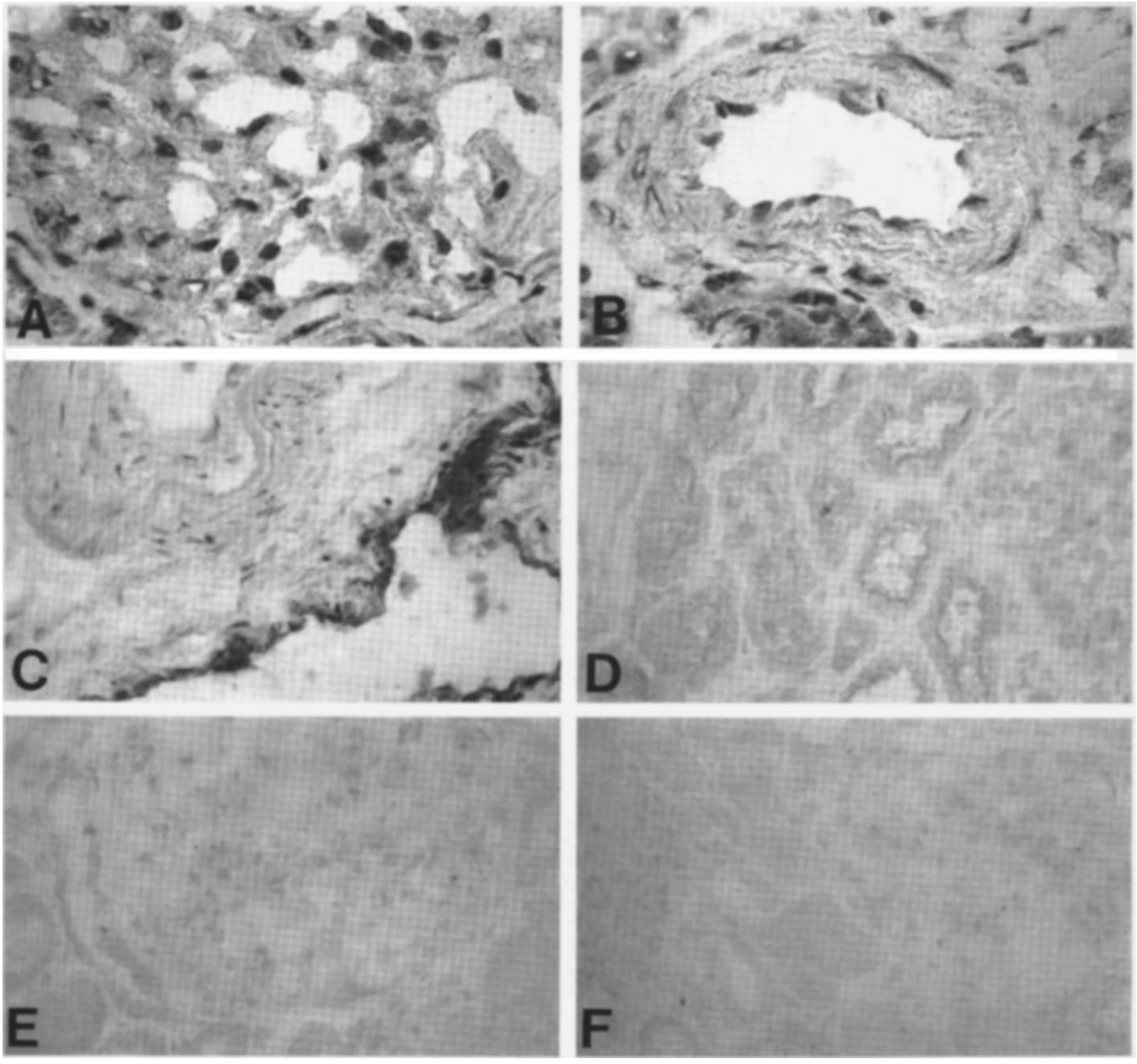

Fig. 2. *Detection of t-PA, u-PA, PAI-1 and uPA-R in normal human kidney by immunohistochemical studies (original magnifications: A, B 10 × 50; C, D, E, F 10 × 25).* In normal human kidney, by APAAP technique a faint endothelial t-PA expression was detected in the glomerulus (**A**), and an intrarenal artery (**B**), but a large amount of t-PA antigen was detected in venous endothelial cells (**C**). u-PA was mainly detected in tubular epithelial cells, predominantly at the brush border of convoluted proximal tubules (**D**). PAI-1 (**E**) and uPA-R (**F**) could not be detected in normal human kidneys.

in cells desquamating in the tubular lumen, but not in glomerular cells. Some interstitial infiltrating cells were also stained by anti-uPA-R antibody in thrombotic microangiopathy and acute tubular necrosis (data not shown). The three different anti-uPA-R antibodies gave a similar pattern in all cases. In contrast, no uPA-R antigen could be detected in the normal human kidneys whatever the antibody used. Only few glomerular and arterial endothelial cells weakly expressed t-PA both in thrombotic microangiopathy and in normal human kidneys. In contrast, t-PA antigen was strongly expressed by venous endothelial cells in these two conditions. In addition, in the renal thrombotic microangiopathy, some infiltrating cells were also labeled by anti-t-PA antibody (data not shown). u-PA antigen was detected in all tubular sections in thrombotic microangiopathy and in normal human kidney. This expression was more pronounced at the brush border of convoluted proximal tubules. In addition, some glomerular podocytes were also faintly stained by anti-u-PA antibody.

In situ hybridization studies

As shown in Figure 3, glomerular and arterial expression of PAI-1 mRNA was detected in thrombotic microangiopathy in three out of four cases studied, whereas PAI-1 mRNA was never

Table 1. Results of immunohistochemical analysis of uPA-R, PAI-1, u-PA and t-PA antigens on renal biopsies

		uPAR			PAI-1			u-PA			tPA			
	Case	G	T	A	G	T	A	G	T	A	G	T	A	V
Normal controls	1	−	−	−	−	−	−	+	+++	−	+	−	+	+++
	2	−	−	−	−	−	−	−	+++	−	−	−	−	++
	3	−	−	−	−	−	−	+	+++	−	−	−	−	++
	4	−	−	−	−	−	−	−	+++	−	+	−	+	+
	5	−	−	−	−	−	−	+	++	−	+	−	−	++
	6	−	−	−	−	−	−	−	++	−	−	−	−	++
	7	−	−	−	−	−	−	−	+++	−	−	−	−	++
Thrombotic microangiopathy	1	+++	+	+	+++	−	+	+	+++	−	−	−	−	
	2	+	−	−	−	−	−	−	+	−	−	−	−	
	3	++	−	−	+	−	−	+	++	−	+	−	−	
	4	+++	++	+	+++	−	+	+	++	−	+	−	−	
	5	+	−	−	+	−	−	+	+++	−	+	−	−	
	6	++	+	++	−	+	−	++	+++	+	+	−	+	
	7	+++	+	++	−	−	+	−	+++	−	+	−	+	
	8	++	−	−	−	−	−	−	+++	−	−	−	−	
	9	+++	++	+	+++	−	−	+	+++	−	++	−	−	
	10	+	−	−	−	−	−	−	+++	−	−	−	−	
Acute tubular necrosis	1	−	++	−	−	−	−		ND			ND		
	2	−	+	−	−	−	−		ND			ND		
	3	−	+	−	−	−	−		ND			ND		
	4	−	+	+	−	−	−		ND			ND		
	5	−	+	+	−	−	−		ND			ND		

The localization of uPA-R, PAI-1, u-PA and t-PA antigens in the different kidney structures is reported: glomeruli (G), tubules (T) and arterioles (A) or veinules (V).

detected in normal human kidneys. Similarly, glomerular expression of uPA-R mRNA was also observed in thrombotic microangiopathy in three out of seven cases studied. In addition, uPA-R mRNA could be detected in some desquamated tubular epithelial cells in correlation with the immunohistochemical findings. These glomerular and tubular uPA-R mRNA expressions were never detected in normal human kidneys. Compared to normal human kidneys, in which a strong and homogeneous expression of t-PA mRNA was found in all glomeruli, we found a decreased glomerular t-PA mRNA expression in thrombotic microangiopathy kidney in four out of six cases. In addition, t-PA mRNA was detected in endothelial cells of renal vessels in both thrombotic microangiopathy and normal human kidneys. By this technique, we did not find t-PA mRNA in any tubular sections. Finally, u-PA mRNA expression was detected only in some tubular sections, in the deep cortex and the outer medulla, corresponding to the straight parts of proximal and distal tubules both in thrombotic microangiopathy and in normal human kidneys. No significant signal was found in the glomeruli and the arterioles nor in proximal and distal convoluted tubules. The specificity of the signals obtained with the different probes was confirmed by the absence of signal when tissue sections were treated with RNase before the incubation with the labeled probe.

Discussion

In the present study, by immunohistochemistry and *in situ* hybridization studies, we investigated the expression of the plasminogen activation system in renal thrombotic microangiopathy as compared to acute tubular necrosis in which tubular, but not glomerular injury is observed and to normal human kidneys. We demonstrate for the first time an intrarenal up-regulation of PAI-1 and uPA-R expression in most of the cases of thrombotic microangiopathy. We also show that no PAI-1 and uPA-R expression can be detected in the normal human kidney. In addition, we found a decreased glomerular t-PA mRNA expression in some cases of thrombotic microangiopathy whereas u-PA expression was not significantly altered as compared to normal human kidneys.

In thrombotic microangiopathy, but not in acute tubular necrosis nor in the normal human kidney, the induction of PAI-1 and uPA-R expression was mainly found in glomeruli and in vascular walls of arterioles with thrombotic and/or proliferative lesions. Interestingly, uPA-R and PAI-1 antigens were detected in almost all the cases, whereas uPA-R mRNA and PAI-1 mRNA were detected in some but not all the cases, suggesting that the up-regulation of uPA-R and PAI-1 mRNA is transient and that the uPA-R and PAI-1 antigens have a longer half-life than the corresponding mRNA. The present study confirms and extends our previous findings [14] showing that the accumulation of PAI-1 in glomeruli and in vascular walls results from the local overexpression of PAI-1 by the resident cells of glomeruli and vascular walls. This excess of PAI-1 could inhibit local t-PA activity and thus promote the glomerular and vascular fibrin accumulation which is observed in thrombotic microangiopathy. t-PA antigen was mainly detected in venous endothelial cells and to a lesser extent in glomeruli and in arterial endothelial cells. In contrast, t-PA mRNA was strongly expressed in glomeruli as well as in all vascular endothelial cells suggesting a rapid release of newly synthetized t-PA from glomeruli and arteriolar endothelial cells. Similar sites of synthesis were reported in the murine kidney [5]. Our results also show a decreased glomerular t-PA mRNA expression in some cases of thrombotic microangiopathy. However, quantitation of the signal obtained by *in situ* hybridization is difficult and direct measurements of t-PA mRNA would be necessary to confirm this result. A decreased t-PA synthesis, combined with an up-regulation of PAI-1 synthesis is likely to induce a decrease in local fibrinolytic activity.

Induction of uPA-R expression in glomerular and vascular

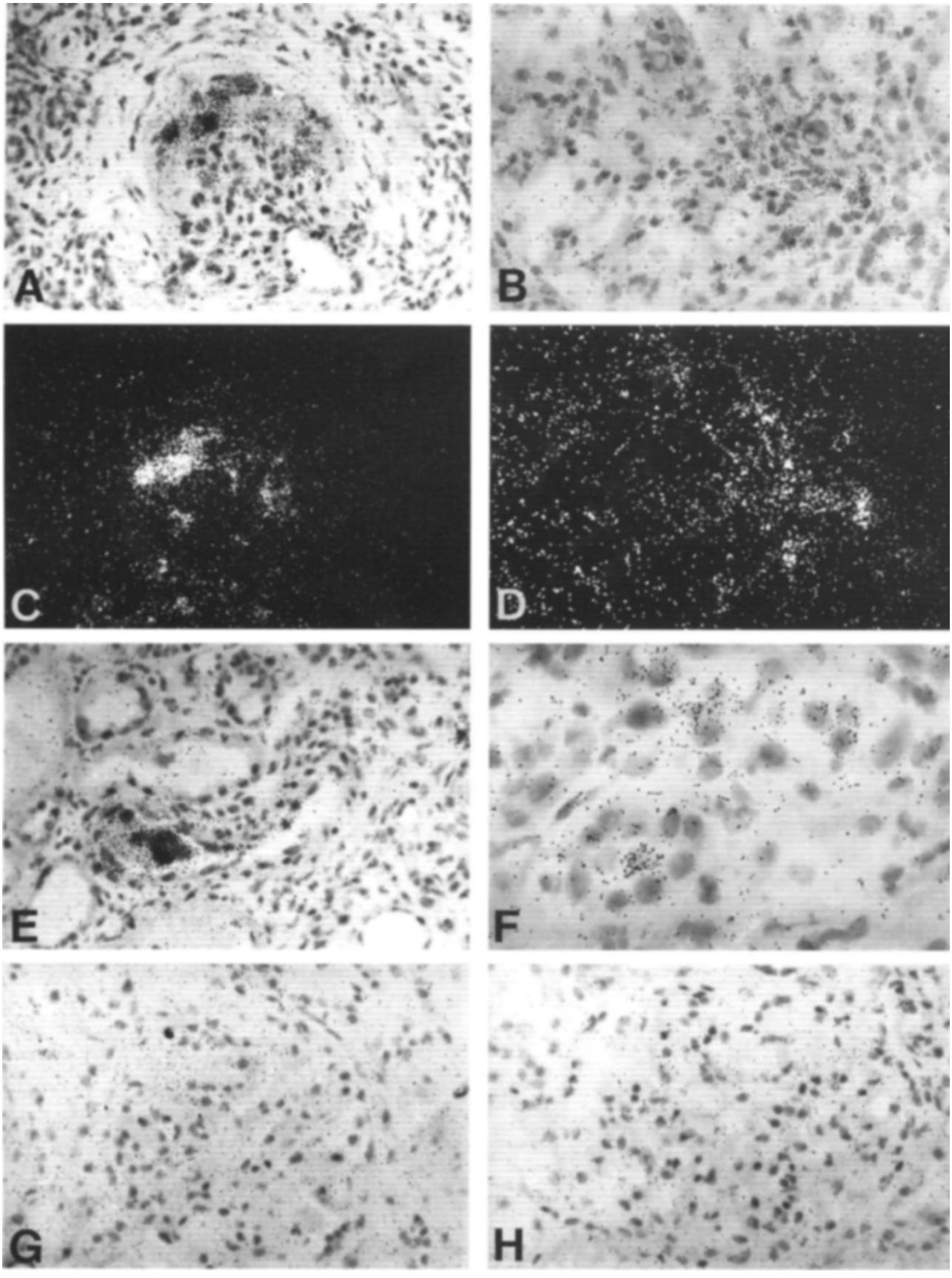

Fig. 3. *Localization of PAI-1 and uPA-R mRNA in renal thrombotic microangiopathy by in situ hybridization (original magnifications: A, B, C, D, E, G, H, 10 × 25; F 10 × 50).* A strong PAI-1 mRNA expression was detected in glomerulus (**A**, bright field; **C**, dark ficld) and in arteries (**E**). A weak glomerular uPA-R mRNA expression was detected in renal thrombotic microangiopathy (**B**, bright field; **D**, dark field). Some tubular epithelial cells were also found to express u-PA-R mRNA (**F**). No specific signal was detected if tissue sections were treated with RNase before the incubation with the ^{35}S-labeled PAI-1 (**G**) or uPA-R (**H**) probe. Only the background signal is observed.

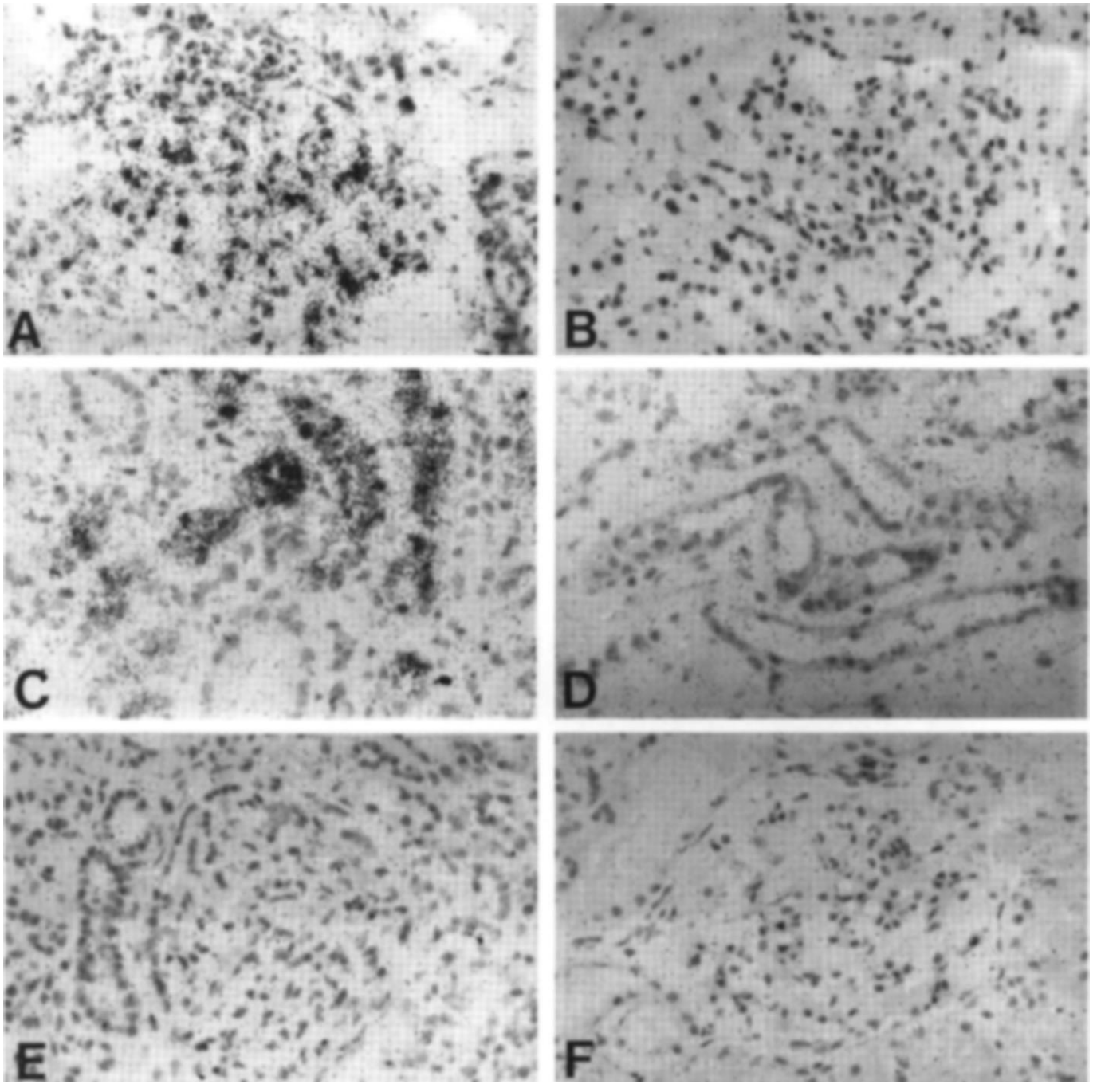

Fig. 4. *Localization of t-PA, u-PA, PAI-1 and uPA-R mRNA in normal human kidneys (original magnification 10 × 25).* A strong glomerular expression of t-PA mRNA was detected (**A**) and the specific signal was suppressed if the tissue sections were pretreated with RNAse before hybridization with the ^{35}S labeled t-PA probe (**B**). u-PA mRNA was detected in tubular epithelial cells in the deep cortex and the outer medulla (**C**) and no specific signal was detected on RNAse pretreated sections (**D**). PAI-1 (**E**) and uPA-R mRNA (**F**) were not detectable in normal human kidneys by in situ hybridization. Only the background signal is observed.

endothelial cells and in tubular epithelial cells as well as in some inflammatory cells in thrombotic microangiopathy indicates an activated phenotype of these cells. *In vivo*, the induction of uPA-R expression has been reported in tumor cells at invasive foci and in tumor-associated macrophages, fibroblasts and endothelial cells [22, 29–31]. It has been recently reported that uPA-R expression, which is undetectable in the normal murine kidney, is up-regulated after endotoxin administration [8], suggesting a role of endotoxin itself or of cytokines such as IL-1 and TNF-α in the induction of u-PA-R expression. The effect of uPA-R expression may be beneficial by promoting cell-surface plasminogen activation and fibrin degradation. Furthermore, uPA-R has been shown to bind uPA-PAI-1 complexes which are then internalized and degraded within the cells [32], suggesting that uPA-R could also enhance the local fibrinolytic activity by promoting PAI-1 degradation. In addition, the cell surface expression of uPA-R may

promote matrix degration, cell adhesion and proliferation [33–35], all processes required for the formation of proliferative and infiltrating lesions [1, 2]. Interestingly, both in thrombotic microangiopathy and in acute tubular necrosis, the presence of uPA on almost all tubular sections and the overexpression of uPA-R without PAI-1 expression in some tubular epithelial cells, particularly at the surface of desquamating cells, may indicate a role of uPA-R and u-PA in the process of cell detachment from the basement membrane. The brush border of proximal tubular epithelial cells was particularly strongly stained with anti-uPA antibodies. In contrast, u-PA mRNA was only found in tubular epithelial cells of the deep cortex and the outer medulla. These latter results are also in agreement with the study of Sappino et al in the murine kidney [5]. The absence of u-PA mRNA in convoluted proximal tubules suggests that u-PA antigen that we detected in these cells is not synthesized *in situ*. A likely explanation may be that u-PA, filtrated throught the glomerulus reaches the proximal tubule and binds to gp330, a 330 kDa glycoprotein that is located at the apical membrane of proximal epithelial cells and is involved in u-PA uptake [36].

In conclusion, in the present study, using immunohistochemistry and *in situ* hybridization methods, we demonstrate an induction of PAI-1 and uPA-R expression in glomeruli and vascular walls during renal thrombotic microangiopathy. Using the same techniques, these molecules could not be detected in normal human kidneys. The induction of PAI-1 expression in glomeruli and arterial walls could play a role in the persistancy of fibrin. The overexpression of uPA-R in glomerular and vascular cells suggests an activated phenotype of these cells, but the exact role of uPA-R in the process of thrombotic microangiopathy remains to be investigated.

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